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FINAL PROGRESS REPORT

Research Project Contract Number: Da 18-035-AMC-143(A)

Biochemistry and Mechanism of Action of Toxic Proteins

Report Period: 30 June 1964 to 30 April 1967

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The following report summarizes the total work completed under Contract DA 18-035-AMC-143(A) through 30 April 1967. Although no longer supported by this contract, the work is currently being continued in the Neurotoxicology Laboratory of Pennsylvania Hospital Subsequent reports on unsupported work will be forthcoming.

Studies have been made on the binding of Botulinus toxin to its presumed sites of action; on the absorption of the toxin from various areas of the gastrointestinal tract; and on those physical and chemical properties of the toxin which are relevant to its mode of action. This final report does not present experimental details but is a summary of the results obtained in the various stages of the investigation. Previous reports submitted during the period of the contract contain detailed accounts of the experiments performed and the results obtained. The last of these reports was submitted up to December of last year and the contract ran for a further 6 weeks. Work carried out in this period will be included in a later report.

Section I deals with an attempt to confirm the specific binding of toxin at the neuromuscular junction by use of fluorescent labelled toxin and antitoxin. Sections III and IV summarize the results of studies in which very small doses of Botulinum A toxin were used to produce either a chronic generalized intoxication or a localized paralysis. In these "mild" cases, the period of intoxication was of the order of days and hence we looked particularly for ultrastructural changes in the neuromuscular junction which might be expected to develop in a long term neuromuscular defect. In section V, we report our studies on the absorption of Botulinum toxin from the gastrointestinal tract. These studies were made on direct visualization of fluorochrome labelled botulinum toxin and we have been able to show that toxin is apparently absorbed from the oropharyngeal cavity.

I. In Vitro Binding of Fluorescein Labelled Botulinus A Toxin and Toxin Fractions.

The major sites of binding of fluorescein or rhodamine labelled botulinus toxin occurred in striated muscle where the sarcolemma was stained and focal patches of fluorescence consistent in size and shape with motor endplates were observed. The central nervous system and viscera failed to bind the toxin under the conditions employed. In small intestine, structures consistent with sympathetic or parasympathetic ganglia were also stained. Labelled fractions prepared from dissociated toxin (M.W. 77,000 to 150,000) showed some staining of central nervous system as well as staining of motor endplates. In indirect experiments utilizing fluorescein labelled botulinus antitoxin, no binding sites were observed in the central nervous system but motor endplates were prominently and selectively stained. Studies performed with ferritin-labelled botulinus toxin fractions showed localization within the subneural apparatus as previously observed with botulinus B toxin.

From these studies, it is clear that there is selective in vitro and in vivo binding of botulinus toxin in motor endplates. However, the limitations of resolution with fluorescence microscopy did not permit us to distinguish a precise site of localization. Work currently in progress utilizing a new method based on peroxidase labelled antitoxin should help us to decide if terminal axon or subneural apparatus is the major site of toxin binding.

II. Effect of Botulinus Toxin on Binding of Electron Dense Cations in the Neuromuscular Junction.

A discovery of Savay and Csillik (1948) that lead was selectively bound in the neuromuscular junction has been confirmed by ourselves and other

investigators. It seems likely that the anionic sites that bind lead in this type of experiment are related to the specific properties of the junctional membranes. In a series of experiments testing the effect of botulinus toxin on the binding of lead to endplates, no alterations were found.

III. Chronic Botulinus Intoxication:

In a series of experiments with groups of mice, minute doses of botulinus toxin were injected by various routes. The animals were observed for up to 20 days during which a syndrome of severe generalized weakness occurred. Muscles from these animals were studied after perfusion with glutaraldehyde in the electron microscope. Even after 16 to 20 days, no changes in the neuromuscular junctions were found although non-specific changes were observed in the mitochondria of the skeletal muscle. These changes are tentatively attributed to anoxia rather than to a specific effect of the toxin. Thus the "chemical denervation" produced by botulinus toxin does not result in degenerative changes in endplates such as those seen after nerve section.

IV. Local Botulism.

In analogy with the situation with tetanus exotoxin, it was found that injection of minute doses (0.005 gamma in 1 ml.) directly into gastrocnemius muscle of mice produced a syndrome of local botulinus intoxication. These animals showed localized paralysis that was maximally developed in 4 to 6 days after injection. By 15 days, recovery of muscle function was observed. Ultrastructure studies of these muscles showed no abnormalities in the motor endplates. This reversible phenomenon is of considerable interest and is worthy of additional investigation.

Resistant Animals:

During the studies with mice, we discovered that in any batch of randomly bred white mice, 5 t 12% of the animals showed a remarkable resistance to botulinus toxin. These animals lived 10 to 20 times longer than the other experimental animals in the group and some survived indefinitely. Breeding through 5 generations of mice which had survived a toxic dose yielded a group of animals large enough to be tested against a control group for the possible presence of acquired resistance. It was found that unlike the original group none of the F-5 generation had total resistance to the toxin although the control group of 100 mice did have members showing resistance. However, the mean death time of the experimental group was slightly higher than the mean death time for the control group when animals surviving more than 10 times the mean were excluded. These result we have previously reported. The possibility arises that a number of genes are responsible for resistance and that inter-breeding has dispersed these genes among the whole group. The possibility of a resistance strain in mice is intriguing and might perhaps be made the object of a future study. These resistant mice would be of considerable value in studying the basic physiology of botulinus intoxication.

V. Absorption of Botulinus Toxin:

In a series of experiments with fluorescein-labelled botulinus toxin, we found that the toxin was rapidly destroyed in the stomach. Absorption could be traced in the oropharynx and proximal small intestine. In the intestine, fluorescein-labelled material was seen between the epithelial cells and in the submucosal lymphatics. There was no evidence of a pinocytotic transport of the protein. Further studies showed that "stomach tube" experiments were frequently invalidated by the marked regurgitation which occurred leading, in our opinion, to significant oropharyngeal absorption. It may be that the feeding habits of intoxicated humans may bear importantly on the degree and severity of

intoxication. This problem has puzzled previous workers because individuals eating apparently comparable amounts of contaminated food have shown marked variation in their sensitivities to intoxication.

VI: Physical and Chemical Experiments:

Under this heading we include experiments of the bioassay of the toxin. Our original intent was to fractionate the crystalline toxin following previous methods reported by other workers and to obtain from these fragments specific molecular units of consistent size and toxicity which could then be used for our studies. The use of polydisperse toxin makes it more difficult to follow the pathway of the molecule in the development of intoxication and makes analogies between the naturally occurring intoxication and the experimental situation difficult.

Considerable time was therefore spent on column chromatography using the various grades of Sephadex and a variety of electrophoretic techniques in order to define a particular molecular unit which we then intended to use experimentally. This attempt was unsuccessful. In brief summary, we found that although any given technique would yield an identical fractionation of the toxin when applied under standard conditions of pH and ionic strength, any further handling of the toxin would alter the relative proportion of the components. From the rate of elution of such components from Sephadex columns, we found that the range of molecular sizes for toxic botulinum A molecules is from perhaps 5 to 10 thousand up to somewhere of the order of 800 thousand to 1 million. Furthermore, these molecular fragments appear to exist in a dynamic equilibrium with each other. Application of immunoelectrophoretic techniques, comparisons of toxicity and U.V. absorption at 280mμ, and electrophoretic separation of fluorochrome labelled toxin fragments (for all of which experimental details have been previously given) led us to the conclusion that not only were these molecular components in a state of dynamic equilibrium but they were also, in their toxic and labelling

different. Ouchtalony plate studies show no
toxins and commercially produced antitoxins
e correct toxin/antitoxin pair.

of botulinum toxin:

say the binding of botulinum toxin to brain
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in the control vessels. Thus, the presence of
to be protective of toxin rather than responsible
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clarify the nature of the loss of toxicity in
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the denaturation, particularly in dilute solutions,
of the toxin on glass surfaces. In a series
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residual charges on the surface of the glass,
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the preparation of solutions for the testing of toxicity since it places a limitation of accuracy on methods employing serial dilutions. Further experiments showed that albumin will prevent the loss of toxicity at all pH's tested but is, of course, most effective at the iso-electric point. We do not find any positive or negative synergistic effects between the toxin and the albumin and believe that it is as effective as gelatin as a "protective" addition to solutions of toxin.